IMMUNOGENICITY OF RECOMBINANT *Mycobacterium smegmatis* EXPRESSING ANTIGEN 85B EPITOPES AS A POTENTIAL TUBERCULOSIS VACCINE CANDIDATE

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AFB	Acid fast bacillus
Ag85B	Antigen 85B
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
Atg	Autophagy related gene
B.C	Before Century
BCG	Bacille Calmette Guerin
CD	Cluster of differentiation
CFU	Colony forming unit
CFP	Culture filtrate protein
CQ	Chloroquine
CR	Complement receptor
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
ESAT-6	6-kDa Early Secreted Antigenic Target
fbp	Fibronection binding protein
γδ	Gamma delta
HIV	Human Immunodeficiency Virus
HLA	Human Leukocytes Antigen

HRP	Horseradish Peroxidase
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IGRAs	Interferon-γ Release Assays
IL	Interleukin
INH	Isoniazid
iNOS	Inducible nitric oxide synthase
kDa	kilo Dalton
Kan	Kanamycin
LM	Lipomannan
LAM	Lipoarabinomannan
LTBI	Latent TB infection
MDR- TB	Multi-drug resistant tuberculosis
MHC	Major histocompatability complex
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
M. obuense	*Mycomacterium obuense*
M. vaccae	*Mycobacterium vaccae*
MycOri	Mycobacteria origin of replication
NAATs	Nucleic acid amplification test
NCDs	Non-communicable disease
NH_4Cl	Ammonium chloride
NK	Natural killer
NO	Nitric oxide
NTM	Non-tuberculous mycobacteria

OD	Optical density
PAMPs	Pathogen associated molecular patterns
PIM	Phosphatidyl-myo-inositol mannosides
PRR	Pathogen recognition receptor
PPD	Purified protein derivative
PZA	Pyrazinamide
QFT-G	Quantiferon TB Gold
QFT-GIT	Quantiferon TB Gold In Tube
rBCG	Recombinant bacilli Calmette Guerin
rMS	Recombinant *Mycobacterium smegmatis*
RD	Region of difference
RIF	Rifampin
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RT	Room temperature
SDS PAGE	Sodium Dedocyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard error mean
TB	Tuberculosis
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TST	Tuberculin skin test
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant-Tuberculosis

KAJIAN KEIMUNAN *Mycobacterium smegmatis* REKOMBINAN YANG MENGEKSPRES ANTIGEN 85B SEBAGAI CALON VAKSIN BERPOTENSI TERHADAP TUBERCULOSIS

ABSTRAK

Penyakit Tuberculosis (TB) masih kekal sebagai penyumbang kepada masalah kesihatan sedunia yang mengakibatkan lebih daripada 1.3 juta kematian setiap tahun. Pembangunan vaksin yang baharu sebagai pengganti kepada vaksin Bacille Calmette Guerin (BCG) atau bertujuan meningkatkan keberkesanannya adalah merupakan matlamat utama yang telah disarankan oleh Pertubuhan Kesihatan Sedunia (WHO). *Mycobacterium smegmatis* (*M. smegmatis*) adalah sejenis mikroorganisma yang tidak patogenik dan merupakan komensal kepada manusia, dan ia mempunyai ciri-ciri yang hampir sepadan dengan *Mycobacterium tuberculosis* (*M. tuberculosis*). Vektor mikobakteria hidup termasuk *M. smegmatis* telah berjaya digunakan sebagai vaksin eksperimentasi terhadap TB. Dalam kajian ini, *Mycobacterium smegmatis* rekombinan yang mengekspresi epitope Ag85B terpilih (P1, P2 dan P3) telah dihasilkan (rMs064). Kesan immunomodulasi oleh rMs064 terhadap sel makrofaj mencit J774A.1 telah dinilai dalam kajian ini. Keputusan dari eksperimen mendapati keupayaan rMs064 untuk merangsang pengekspresian penanda pengaktifan makrofaj; MHC kelas II dan molekul CD40 dan merangsang penghasilan sitokin IL-6, IL-10 dan IL-12p70. Kedua-dua strain rMs064 dan *M. smegmatis* yang di transformasikan dengan plasmid kosong (rMs012 sebagai kawalan) juga berupaya mempengaruhi aktiviti fagosit dan mengaktifkan fungsi bakterisid oleh sel makrofaj, merembeskan penghasilan enzim “inducible nitric oxide synthase” (iNOS) dan penghasilan nitrik oksida (NO). Tindakbalas yang dinyatakan merupakan bahan

perantara yang penting untuk aktiviti anti-mikobakteria oleh sel makrofaj mencit. Kami juga menentukan aktiviti autofagi dengan kuantifikasi protein LC3B-II pada sel makrofaj yang telah dijangkiti rMs064. Walaubagaimanapun, keputusan mendapati aktiviti autofagi dalam sel makrofaj yang telah di jangkiti oleh rMs064 adalah tidak konklusif dalam kondisi eksperimen kami. Seterusnya, rMs064, rMs012 dan PBS (kawalan) telah diimunisasi kepada mencit Balb/C untuk kajian keimunan. Penentuan keupayaan rMs064 untuk mengaruh penghasilan tindakbalas sel dan humoral terhadap epitop spesifik yang diekspres telah dijalankan. Kami mendapati proliferasi signifikan sel limpa yang diasingkan dari mencit yang telah diimuninasi dengan rMs064 memberikan tindakbalas yang spesifik terhadap epitope P2 dan P3, berbanding kawalan. Penghasilan sitokin dan immunoglobulin G (IgG) total serta subkelas IgG juga ditentukan terhadap mencit yang telah diimunisasi dalam kajian ini. Penghasilan sitokin IFN-γ adalah signifikan hanya pada sel limpa dari kumpulan mencit yang diimunisasi dengan rMs064 yang di aruh oleh epitop P1, apabila dibandingkan dengan kedua-dua kumpulan mencit kawalan. Untuk penentuan tahap pengaktifan sel T oleh rMs064, mencit telah diimunisasi dengan rMs064, rMs012, PBS dan KLH-85B. Sel makrofaj yang telah dijangkiti rMs064 dan di ko-kultur dengan sel limpa yang telah disensitasikan dengan KLH-85B didapati merangsang penghasilan sitokin IFN-γ dan TNF-α yang signifikan. Penghasilan IgG total dan subkelas IgG1 menunjukkan peningkatan yang signifikan dalam serum kumpulan mencit yang diimunisasi dengan rMs064 yang diaruh oleh epitop P1, P2 dan P3, berbanding kumpulan mencit kawalan. Walaubagaimanapun, serum daripada kumpulan mencit yang diimunisasi dengan rMs064 berupaya meningkatkan penghasilan subkelas IgG2a dan IgG2b yang signifikan apabila diaruh oleh epitop P2. Keputusan kajian keimunan menunjukkan mencit yang di imunisasi dengan

rMs064 berupaya menghasilkan tindak balas cenderung kepada jenis keimunan Th1. Sebagai kesimpulan, keputusan eksperimen modulasi keimunan secara *in vitro* di dalam sel makrofaj mencit J774A.1 dan kajian tindakbalas keimunan pada mencit yang diimunisasi dengan rMs064 menunjukkan potensinya sebagai calon vaksin TB untuk eksperimen cabaran pada masa hadapan.

IMMUNOGENICITY OF RECOMBINANT *Mycobacterium smegmatis* EXPRESSING ANTIGEN 85B EPITOPES AS A POTENTIAL TUBERCULOSIS VACCINE CANDIDATE

ABSTRACT

Tuberculosis (TB) remains a major worldwide health problem which causes more than 1.3 million deaths annually. The development of a new vaccine as a replacement of Bacille Calmette Guerin (BCG) or to improve its efficacy is one of the goals mooted by the World Health Organization (WHO) to control TB. *Mycobacterium smegmatis* (*M. smegmatis*) is nonpathogenic and commensal in humans, which shares many characteristics with *Mycobacterium tuberculosis* (*M. tuberculosis*). Mycobacterial vectors including *M. smegmatis* have been successfully used in the development of experimental vaccines against TB. In the current study, recombinant *M. smegmatis* expressing selected Ag85B epitopes (P1, P2 and P3) was constructed (rMs064). The immunomodulatory effects of rMs064 were evaluated in J774A.1 murine macrophage cells. Our results demonstrated the capacity of rMs064 to induce the expression of the macrophage activation markers; MHC class II and CD40 molecules, and the production of IL-6, IL-10 and IL-12p70 cytokines. Both rMs064 and *M. smegmatis* transformed with the empty plasmid (rMs012 as a control) were capable to induce phagocytic and mycobactericidal activity in macrophages as well as the production of IL-1β and TNF-α cytokines. In addition, J774A.1 macrophages infected with both rMs064 and rMs012 also increased the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO), which are crucial mediators of anti-mycobacterial activity. The induction of autophagy as quantified by LC3B-II detection in rMs064 infected macrophages showed inconclusive results in our experimental conditions. The capacity of rMs064 to

induce specific cellular and humoral immune responses in Balb/C mice against the expressed epitopes was evaluated. The proliferative responses of splenocytes from rMs064-immunized mice stimulated with P2 and P3 epitopes showed significant increase as compared to control groups. Cytokine production by splenocytes upon stimulation with Ag85B epitopes and serum specific IgG and its subclasses were determined. The results showed significant production of IL-12p70 and IL-23 when splenocytes were stimulated with P1, P2 and P3 epitopes in mice immunized with rMs064. In addition, significant IFN-γ production was found in splenocytes of rMs064-immunized mice when stimulated only with P1 epitope. For the evaluation of the T cell activation by rMs064, mice were immunized with rMs064, rMs012, PBS and KLH-85B. Macrophages infected with rMs064 and co-cultured with KLH-85B sensitized splenocytes induced significant production of IFN-γ and TNF-α. The specific total IgG and IgG1 subclass showed significant increase against all three Ag85B epitopes on rMs064-immunized mice as compared to controls. rMs064 immunized mice showed significant increase of IgG2a and IgG2b only against P2 epitope. Results of cellular and humoral immunogenicity showed the induction of potent Th1 type immune responses in rMs064 immunized mice. Taken together, the results of *in vitro* immunomodulatory effects and immunogenicity support the future evaluation of rMs064 as an experimental vaccine against TB in challenge experiments.

CHAPTER 1

INTRODUCTION

1.1 General history of tuberculosis

Over the centuries, the world has recorded a tremendous impact in the discovery and the evolution of tuberculosis (TB). TB has been present in humans since ancient times throughout known history and human prehistory. The scientific discovery of TB has been documented in China, India and Egypt at around 2, 300, 3, 300 and 5, 000 years ago, respectively (reviewed by Zink et al., 2003). Evidence for the presence of *M. tuberculosis* complex has been recorded in ancient skeletal and mummified materials as early as 2125 BC (Donoghue et al., 2009) . The DNA analysis of tissue samples from Egyptian mummies has shown that the original *M. tuberculosis* complex was similar to the present *M. tuberculosis* strains (Baron et al., 1996).

M. tuberculosis was first identified in 1882 when Robert Koch (1843-1910) succeeded in visualizing and described that the tubercle bacillus as aetiologic agent of TB (reviewed by Cambau and Drancourt, 2014). Later in 1896, the *Mycobacterium* genus was included into the Mycobacteriaceae family by Lehman and Neuman (Ostroff, 2008).

Since the past few decades, TB has emerged as a major global threat and efforts have been focused to reduce TB disease burden. The Stop TB Strategy mooted by WHO have leaded a serious effort by global scientists in the field to

reduce the prevalence of TB and the mortality associated with the disease. One of the main components of the Stop TB Strategy in the context of the Millenium Development Goals (MDGs) towards reduction of TB burden has been directed to the continuous research for the identification of new anti-TB drugs and biomarkers. The development of new vaccines and diagnostic tools are part of the main efforts to eradicate the overwhelming threat of TB.

1.2 Epidemiology of tuberculosis

TB remains a major global scourge. It infects millions of people each year and causes significant public health challenges especially in developing countries. It is estimated that one-third of the world's population is infected with *M. tuberculosis*. However, the vast majority of infected individuals are protected by the host's immune system and not showing any symptoms of active TB disease. As a consequence, these individuals are healthy but remain latently infected with *M. tuberculosis*. About 5-10 % of newly or latently infected individuals develop TB disease which results in unacceptable number of annual death tolls (WHO, 2011).

The extent of TB epidemics can be classified as high prevalence and low prevalence areas in which the burden and rates of TB are variably distributed throughout the world. The high prevalence areas are countries that suffer TB epidemics with an incidence rate of more than 100 per 100, 000 population. These are mostly countries in Africa with the highest rates per capita while developing countries in Asia show the highest number of TB cases (Figure 1.1).

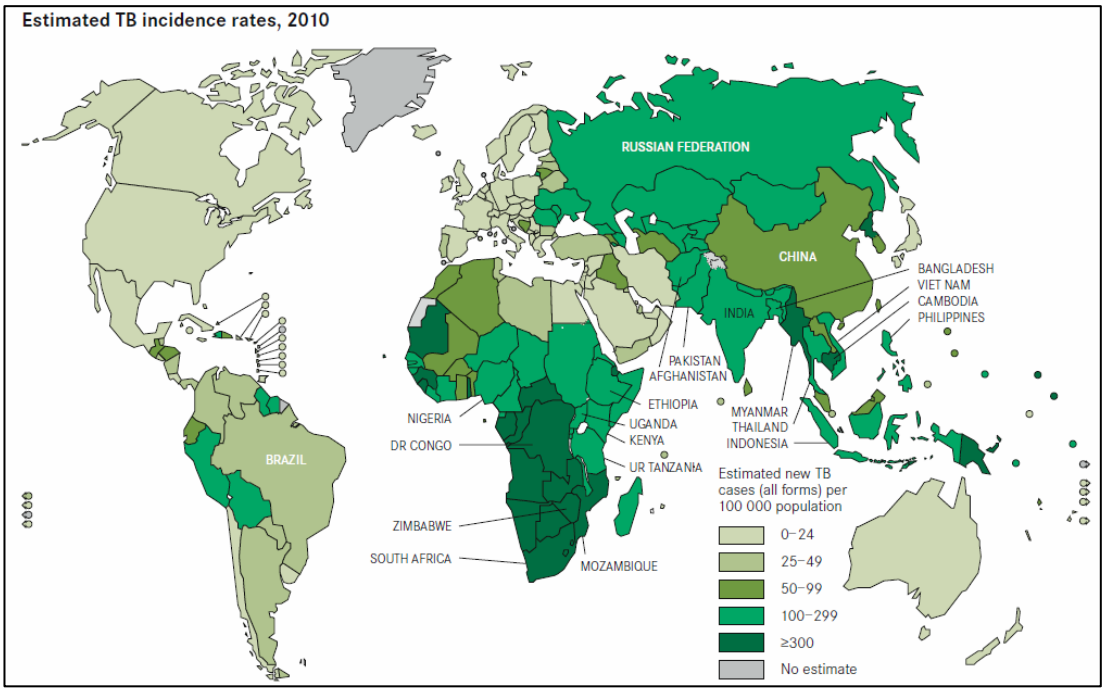

Figure 1.1: Estimated TB incidence rates by different regions and countries, 2010. (WHO, 2011)

In 2014, WHO estimated that 14 million people have active TB. There were about 1.5 million deaths due to TB including 0.4 million co-infected with the human immunodeficiency virus (HIV) (WHO, 2011). Numerous factors have been associated with the increase in global TB incidence which includes the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis*, immigration from high TB prevalence areas and increased number of immune compromised patients (reviewed by Millet et al., 2010). HIV-induced immunosuppression greatly enhances the reactivation of TB disease due to weakened immune responses (Selwyn et al., 1989; WHO, 2011) . However since 2006, a steep decline in TB incidence has been recorded as a result of global efforts in TB control by the comprehensive implementation of Directly Observed Therapy Strategy (DOTS). Several remarkable successes in TB control have been recorded after the implementation of DOTS. The most significant achievement of DOTS between 1995 and 2010, is represented by the fact that almost 55 million active TB patients were successfully treated under continuous surveillance by the national control programmes using the WHO-promoted plan (reviewed by Hill and Whalen, 2014).

1.3 The organism: *Mycobacterium tuberculosis*

Mycobacteria belong to the family of Mycobacteriaceae and the order Actinomycetes. *M. tuberculosis* is a non-motile, straight or slightly curved rod-shaped and non-spore forming bacterium. The mycobacteria size is approximately 1-4 μm in length and between 0.2-0.6 μm in width, making them smaller than most bacterial pathogens. These organisms usually contain granules and vacuoles but they

do not form capsules, flagella or spores. *M. tuberculosis* is an obligate aerobe and intracellular bacterium, and has a slow generation time between 15 to 20 hours.

Several species of mycobacteria, with almost identical growth characteristics and biochemical reactivity are classified into the *M. tuberculosis* complex (MTBC). The MTBC includes *M. bovis*, *M. africanum*, *M. canetti* and *M. microti* which also caused TB in mammals. *M. bovis* is the causative agent of bovine TB and infects mammalian species including humans (Ayele et al., 2004). Among the large spectrum of mycobacterial species, *M. tuberculosis* is the main aetiologic agent of TB in humans.

The cell wall of *M. tuberculosis* is unique and contains lipid-rich component compared to other prokaryotes. The major *M. tuberculosis* cell wall component is lipid which constitutes more than 60% of the total mass and gives unique characteristic to the bacterium. In general, the mycobacterial cell wall consists of a highly structured lipid component known as mycolyl-arabinogalactan-peptidoglycan (mAGP) complex. The mAGP complex, mycolic acids and extractable lipids formed the mycobacterial outer membrane. The mycolic acid provides a hydrophobic layer for intercalating additional complex lipids, which make the mycobacteria highly resistant for the penetration of antibiotics (Minnikin et al., 2002) (Figure 1.2)

Other type of lipids and glycolipids found in mycobacteria cell-wall include phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) (Brennan & Nikaido, 1995; Morita et al., 2004). These complex glycoconjugates are the main virulent components in modulating the host-

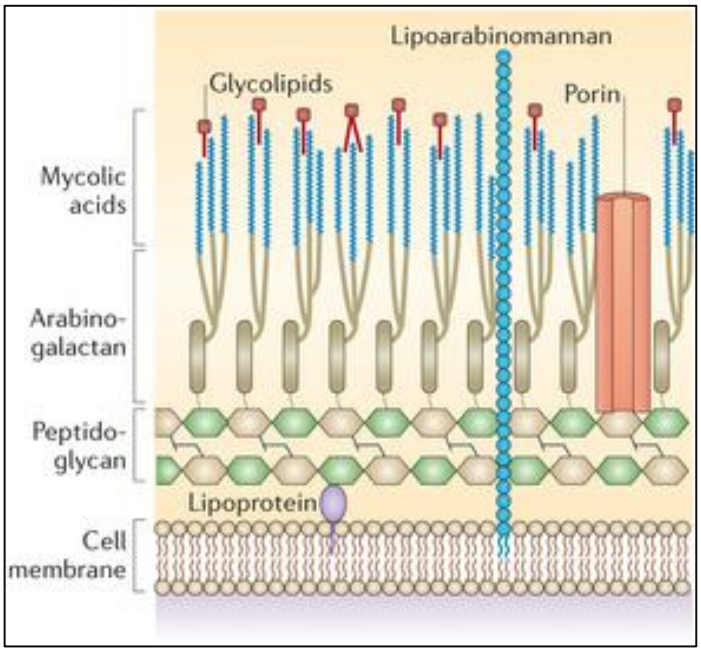

Figure 1.2: Structure of mycobacteria cell wall (Brown et al., 2015).

response during infection. PIMs, LM and LAM have several immunomodulatory features by mediating the interaction with different receptors of the immune system (Schlesinger et al., 2008). LAM and related glycoconjugates are unique across species as the biochemical analysis of the mycobacterial cell wall show different variants of lipids (Mishra et al., 2011). For example, in *M. tuberculosis* and other pathogenic mycobacteria, the end-product of LAM biosynthesis is different from that of non-pathogenic *M. smegmatis* (Figure 1.3). This suggests a different degree of virulence among mycobacterial species which is associated with the biosynthesis of cell wall lipids (Mishra et al., 2011).

1.4 Pathophysiology of tuberculosis

TB is an air borne disease and is acquired when a person inhales droplet containing *M. tuberculosis.* The droplet is a particle containing the tubercle bacilli, and this very fine particle pass through the nasal passages to reach the alveoli of the lungs (Kaufmann, 2002).

The pathogenesis of *M. tuberculosis* infection can be characterized into three different but interrelated interactions between the pathogen and host (Figure 1.4). The first stage is the entrance of *M. tuberculosis* into the lungs where the inhaled bacteria penetrate into the terminal alveoli. After inhalation, particles containing one to three bacilli reaches the lungs where they are phagocytosed by immune cells: alveolar macrophages (AM) and dendritic cells (DC) (Cooper, 2009). Usually most of the internalized bacilli are destroyed at this stage; however, the bactericidal capacity of the AM is not clear (van Crevel et al., 2003).

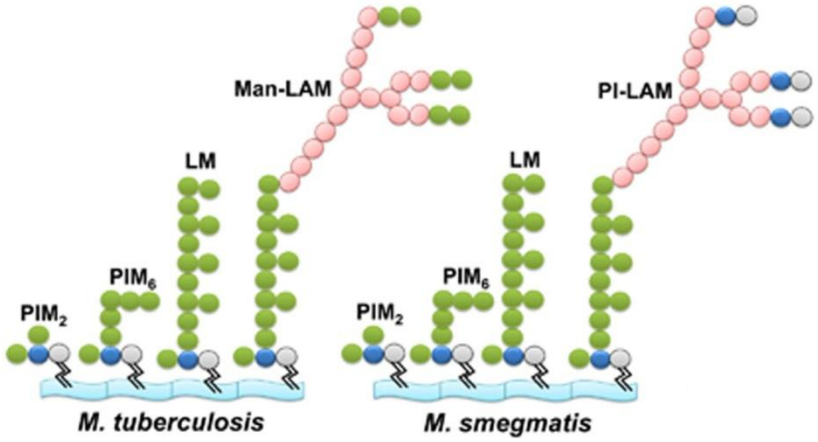

Figure 1.3: The similarities and differences of the cell wall structure of *M. tuberculosis* and *M. smegmatis* (Mishra et al., 2011).

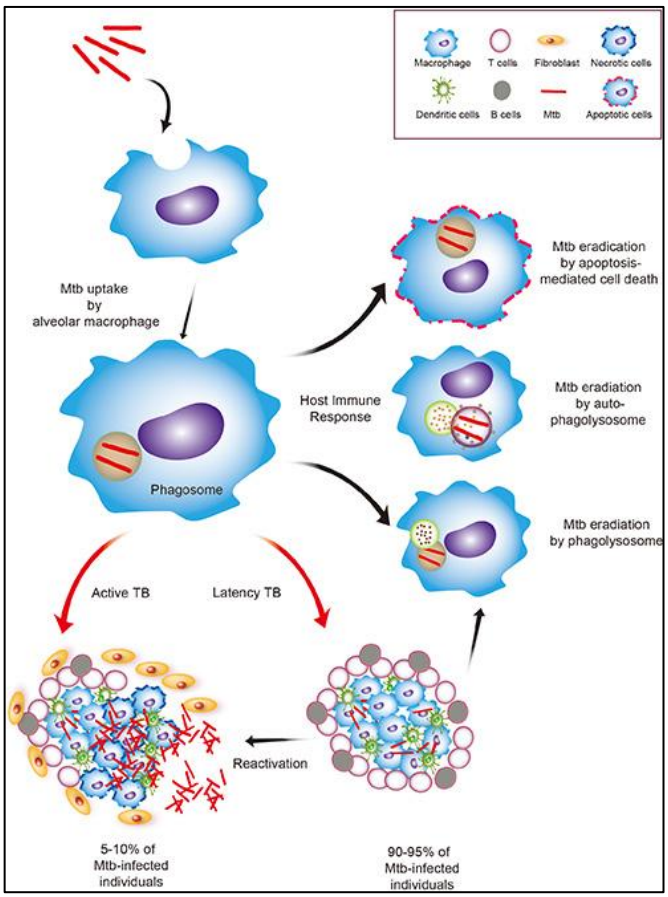

Figure 1.4: A schematic overview of host immune response against *M. tuberculosis* infection (Yuk and Jo, 2014).

The bacilli that evade the initial elimination will multiply in a hostile macrophage environment and *M. tuberculosis* has evolved various mechanisms to ensure their survival in AM. The survival mechanisms used by *M. tuberculosis* include the inhibition of the reactive oxygen and nitrogen intermediates production (ROIs and RNIs, respectively) inhibition of the phagosomal maturation by reducing its acidification and induction of anti-inflammatory responses (Flynn, 2006; Cooper, 2009; Welin et al., 2011).

If the *M. tuberculosis* growth is arrested at early stage of infection, then the disease is maintained in latent condition without TB symptoms in 90-95% of infected individuals. Otherwise, in 5-10 % *M. tuberculosis* infected individuals, the bacilli replicate into macrophages and disseminated to other tissues and organs, where active TB develops with typical clinical symptoms including weight loss, chest pain, frequent coughing and fatigue (Ahmad, 2011).

The second stage of infection is the establishment of cell mediated immune response and the formation of granulomas. In most cases, tubercle bacilli that escape the bactericidal effect of AM will establish the infection and result in destruction of AMs. This will in turn leads to a chemotactic response that brings monocyte-derived DCs and other inflammatory cells such as neutrophils to the area of infected lungs (Schreiber et al., 2010). At this stage *M. tuberculosis* grows logarithmically with limited tissue damage. Activated antigen-specific T lymphocytes that migrate to the site of infection will proliferate forming an early lesion or tubercle, where macrophages become activated to kill intracellular *M. tuberculosis* (Ulrichs and

Kaufmann, 2006). This phase of infection with continuing T cell activation leads to formation of granulomas where the growth of mycobacteria is put in checks which mark the persistent stage of infection or a phenomenon called as latency (Ahmad, 2011)(Figure 1.5).

The final stage is the reactivation of latent infection into active TB disease. Reactivation of latent *M. tuberculosis* infection can occur months or years afterwards when the immune system fails to contain the bacillary growth in conditions of impaired immune response. This stage requires dormant *M. tuberculosis* cells to exit the phase of latency. There are several reasons that can trigger a reactivation event to occur, and this typically involves the failure to maintain immune competency due to a weakening immune system. Under these conditions, the intact granuloma is structurally disrupted resulting in lung cavitation that leads to appearance of pulmonary disease. Impaired immune function as a result of HIV infection is the most important risk factor for progression to active disease, where a host becomes immuno compromised due to depletion of $CD4^+$ T cells and dysfunctional $CD4^+$ and $CD8^+$ T cells which play important role in protection against TB (Wells et al., 2007).

In latent TB infection, *M. tuberculosis* continues to survive within the intact granulomas but there are no signs or symptoms of TB. Latently infected individuals cannot spread the bacteria to the environment or other people, but are at risk of developing active TB disease. Active TB patients exhibit signs and symptoms of the disease and have a high probability to spread the bacteria to others (Ahmad, 2011).

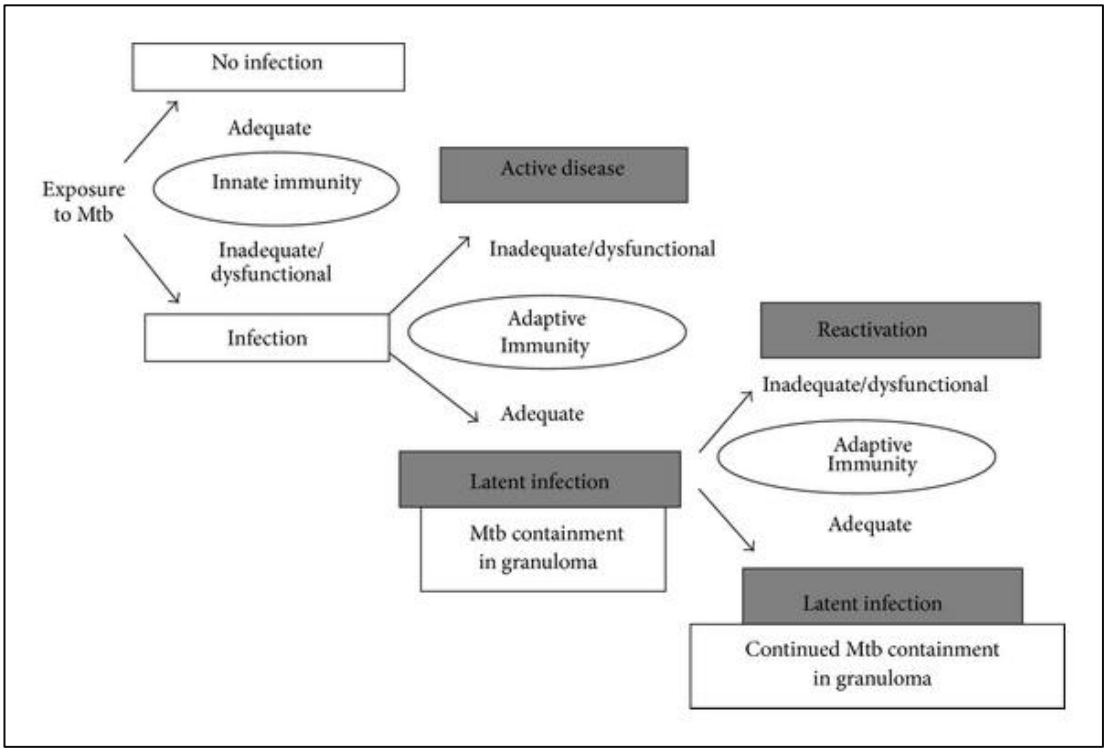

Figure 1.5: Pathogenesis and transmission profile of *M. tuberculosis* (Hossain and Norazmi, 2013).

Functional innate immunity is capable to protect the host from *M. tuberculosis* infection in the majority of individuals, while impaired innate immune response leads to *M. tuberculosis* infection. Activated adaptive immunity then controls the intracellular growth of *M. tuberculosis.* The formation of granulomas which contain persistent *M. tuberculosis* surrounded by immune cells marked the state of latent TB infection. Defective adaptive immunity causes active TB and promotes reactivation of the latent TB.

TB disease can be categorized into two main types, according to site of *M. tuberculosis* infection in the body and where the infection manifests itself. The two types are described as pulmonary and extrapulmonary TB. Pulmonary TB is the contagious form of the disease and accounts for 80-85 % of cases which represents the highest prevalence of death due to TB. Extrapulmonary TB is a result of disseminated *M. tuberculosis* infection to various adjacent organs from the lungs. This form of TB can spread to many different parts of the body through the bloodstream including meninges, peritoneum, bones, lymph nodes, pleura, spinal and spine. Extrapulmonary TB is commonly detected in TB patients co-infected with HIV (Gray and Cohn, 2013).

1.5 Diagnosis and therapy of tuberculosis

The Global Plan to Stop TB was launched in 2006 by the STOP TB Partnership, which set a global blueprint strategy for substantially minimizing TB burden by 2015. The research and development (R&D) component of the Global Plan to Stop TB envisaged that fundamental basic research will pave the way for discovery of new diagnostics, drugs and vaccines (WHO, 2010) . In addition, TB infection control was established by a combination of measures aimed at reducing the risk of TB transmission within population (Mwaba et al., 2011). The DOTS programme was successfully expanded as the principal TB control strategy, focusing primarily on detection and effective treatment of TB cases (Lawn and Zumla, 2011).

1.5.1 Diagnosis

The standard technique used in TB diagnostic laboratory is microscopic examination of acid fast bacillus, followed by culture on solid or liquid media for confirmation. Conventional smear microscopy based on Ziehl-Neelsen staining of sputum specimens with light microscope has been applied for over 100 years in low-resource settings. Microscopy technique helps in the initial diagnosis of TB especially in cases of active infection detection. However, the light microscopy technique is relatively insensitive for the diagnosis of TB as it can only detect the AFB concentration of at least 10^5 AFB per mL of specimen and only detects about 60-70 % of TB cases. Alternatively, fluorescence microscopy technique is 10% more sensitive since the fluorescent bacilli of *M. tuberculosis* can be seen at lower magnification (Steingart et al., 2006).

The isolation of *M. tuberculosis* on a culture medium remains as a gold standard test for TB diagnosis. In this regard, active TB infection is definitive when the organism is directly isolated from human specimens using microbiological culture (WHO, 2010). The most commonly used solid medium is the egg-based Lowenstein Jensen (LJ) medium and a broth based growth system, the BACTEC 460. Liquid medium has been reported to be more efficient and possess 20 % increased sensitivity for the growth and reduced the delay for detection of *M. tuberculosis*. A drawback of the solid LJ culture method is that the result of *M. tuberculosis* culture positivity can be obtained only after 2 to 4 weeks or even longer, and in 10-20 % of cases the bacillus is not successfully cultured. In contrast, the average time for *M. tuberculosis* detection with BACTEC 460 is 15 days and

BACTEC MGIT960 system require 13 days of culture (Huang et al., 2001; Cruciani et al., 2004). However, the contamination rate of liquid medium seems to be higher in comparison with the solid media (Somoskovi et al., 2000). Safety issues concerning the radioactivity of BACTEC and the cost of waste disposal management prevents its widespread application.

The most important molecular test used for laboratory diagnosis of pulmonary TB is the nucleic acid amplification test (NAAT). NAAT is performed following microscopy to distinguish *M. tuberculosis* from other smear-positive mycobacteria. In general, the most common NAAT method used is polymerase chain reaction (PCR). The test is usually performed on sputum or broncho alveolar lavage (BAL) specimens, to detect small amounts of genetic material (DNA or RNA target gene sequence) from *M. tuberculosis* by repetitive amplification of the target sequences.

The main immune mechanism against *M. tuberculosis* infection is based on the mycobacteria-specific responses of T-cells that can be determined by cellular assays such as tuberculin skin test (TST) and IFN-γ release assay (IGRA). TST is an *in vivo* test based on the intradermal administration of a sterile supernatant derived from *M. tuberculosis*-cultured filtrate protein known as purified protein derivatives (PPD). The exposure to PPD results in a delayed-type hypersensitivity reaction, and the diameter of local skin induration is then measured 48-72 h after antigen injection (Sokal, 1975). The low specificity in TST is due to exposure to BCG vaccination and previous exposure to environmental mycobacteria resulting in false-positive results. The drawbacks of TST procedure has been highlighted, among others are due to

inconsistent inoculation of tuberculin and problem associated with the reading of the test as it is operator-dependent and requires the patient to return to the health care centre for result interpretation (Lange and Mori, 2010). Thus, IGRA has been developed trying overcome the disadvantages of the TST.

IGRA is based on the detection of two specific gene products from *M. tuberculosis*: culture filtrate protein 10 (CFP-10) and 6-kDa early secreted antigenic target (ESAT-6). These genes codify immmunodominant antigens that are absent in BCG and most environmental mycobacteria, decreasing the possibility to elicit false positive responses (Harboe et al., 1996). Two types of commercially available IGRA have been developed based on the measurement of IFN-γ production using ELISA, known as QuantiFERON® TB Gold and the latter version of QuantiFERON® TB-Gold In Tube (QFT-GIT) (Cellestis Ltd, Carnegie, Australia). Another commercialized IGRA, T-SPOT TB is based on the counts of the cells producing IFN-γ visualized as spots with the enzyme-linked immunospot (ELISPOT) method (Oxford Immunotec Ltd, Abingdon, UK) (Mori, 2009).

IGRA specificity is higher and superior compared to TST. Although IGRA is unable to distinguish between active TB and LTBI, IGRA results are not confounded by BCG vaccination as well as exposure to NTM. Therefore, IGRA has an important advantage over TST for the determination of human immune response to *M. tuberculosis*, especially in the population with high exposure of environmental mycobacteria.

1.5.2 Therapy

TB can be treated by chemotherapy and the aim of this treatment is to render the patient non-infectious by elimination of all mycobacteria, thus preventing spread of the disease. In addition, the objective of an effective treatment is to prevent generation and transmission of resistant mycobacteria. Implementation of DOTS in TB treatment results in a major improvement in global TB control. In Malaysia, treatment of TB is based on the Clinical Practice Guidelines for the Control and Management of TB, endorsed by the Ministry of Health, Malaysia. TB treatment regime involve two phases; intensive and continuation phase, respectively. The outcome of intensive phase treatment could be observed by rapid sputum conversion and improving clinical condition. While during the continuation phase the treatment will eliminate the remaining bacilli and minimize the chance for relapse.

There are a number of anti-TB drugs available including p-aminosalicylic acid (PAS), Isoniazid (INH), Cycloserine, Kanamycin, Pyrazinamide (PZA), Rifampicin (RIF) and Ethambutol (ETH). The usual standard drug regimen of short course chemotherapy consists of 2 months of INH, RIF, PZA and ETH, followed by 4 months of INH and RIF (CDC, 2014). The patients' compliance to the prescribed treatment regimen is a pre-requisite for successful treatment. TB therapy is relatively cheap but the long treatment regimens often leads to non-compliance and hence results in development of MDR-TB strains (Espinal, 2003). Furthermore, interruption of the antibiotic course may have serious consequences where TB cannot be cured or *M. tuberculosis* can spread to others (Goble et al., 1993). Thus in

addition to developing more effective drugs that require much shorter treatment regimens, other effective strategies such as vaccines needs to be developed.

1.6 The host immune response to *Mycobacterium tuberculosis*

The immune system involves recognition and discrimination of foreign agents as not part of the body and the development of an immune response against them. Defence against foreign invaders or antigens, such as microbes are mediated by initial interaction with innate immune cells and the later responses of adaptive immunity.

Innate immunity provides the first line of defence against intracellular bacterial infection, such as *M. tuberculosis*. The later response for mycobactericidal processes mainly depends on the synergistic interaction between infected macrophages and T lymphocytes. The pivotal roles of innate immunity during the early phase of infection substantially influence adaptive immunity. The professional antigen presenting cells such as macrophages and DCs are the main host cells involved in the innate immune response to mycobacteria, and play a critical role in the initiation of adaptive immunity through various mechanisms and signaling events (van Crevel et al., 2003). *M. tuberculosis* infection induced both humoral and cell-mediated immune responses through a complex interaction between the pathogen and infected host. In this context, it is clear that innate and adaptive immunity are closely connected.

1.6.1 Innate immune response to *Mycobacterium tuberculosis*

Early after inhalation of *M. tuberculosis,* alveolar macrophages are the primary cell type involved in the initial uptake and phagocytosis of the bacilli. After this first encounter, monocyte-derived macrophages and DCs modulated the phagocytic process. The infection process is initiated after recognition of pathogen-associated molecular patterns (PAMP) of *M. tuberculosis* by specific pathogen recognition receptors (PRR) expressed on phagocytic cells. This pathogen-host interaction is central in the initiation and coordination of the host innate immune response that determines the fate of ingested *M. tuberculosis* (Akira et al., 2006). On the host side, phagocytic antigen presenting cells are equipped with specific PRRs capable of recognizing *M. tuberculosis*.

Prior to phagocytosis, *M. tuberculosis* or mycobacterial products are recognized by host PRRs, such as Toll-like receptors (TLR), complement receptors (CR), nucleotide binding oligomerization domain (NOD) like receptors (NLR), and C-type lectins family. The C-type lectins include the mannose receptor, the dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) and Dectin-1 (El-Etr and Cirillo, 2001; Jo, 2008; Harding and Boom, 2010). TLRs are the best studied innate immune receptors involved in recognition of PAMPs of pathogens. In mycobacterial infection, interaction of TLRs ligands with the PAMPs expressed by mycobacteria leads to macrophage activation and triggers downstream intracellular signaling cascades (Figure 1.6).

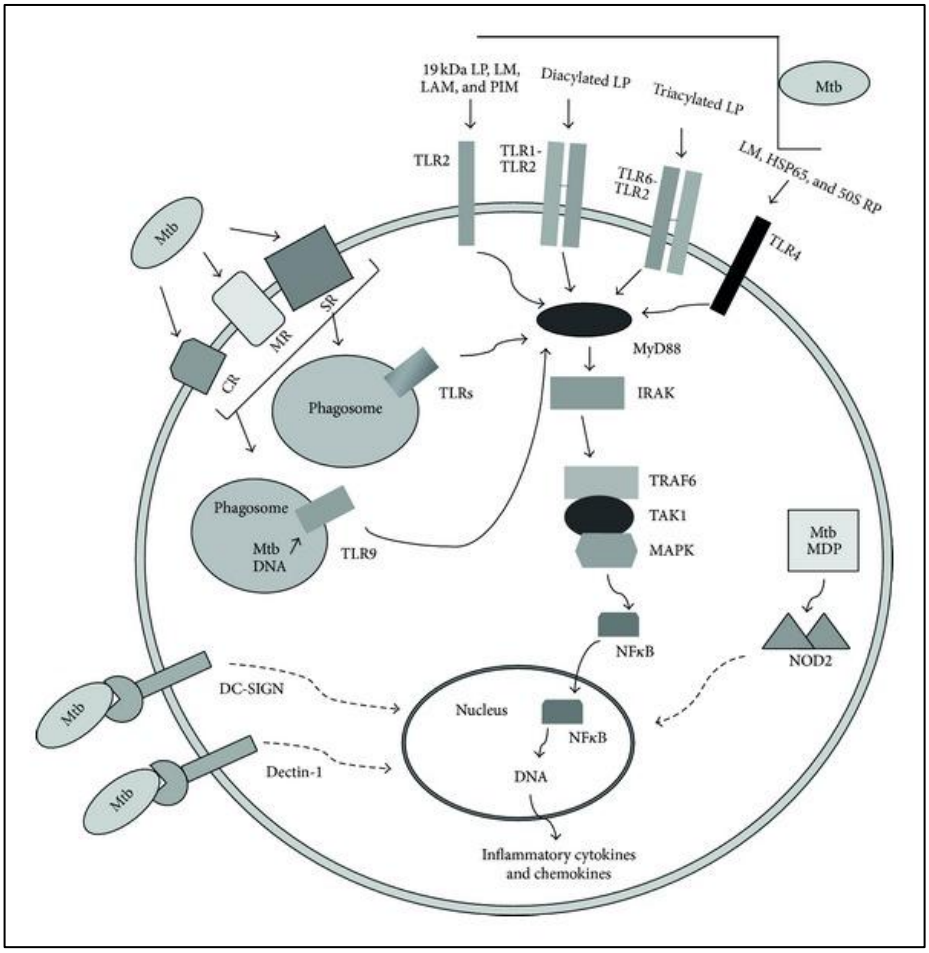

Figure 1.6: Recognition of *M. tuberculosis* or its components by the host's immune receptor during phagocytosis (Hossain and Norazmi, 2013).

Activated macrophages are involved in the regulation of several aspects of the immune response such as the production of proinflammatory cytokines and chemokines, antimicrobial metabolites to kill infected cells, and antigen presentation that bridged the adaptive immunity (Figure 1.7) (Kleinnijenhuis et al., 2011). Important pro-inflammatory innate cytokines produced by activated macrophages includes TNF-α, IFN-γ, IL-1β, IL-18 and IL-12 (Sato et al., 2002; Sharma et al., 2007). TNF-α is secreted by infected macrophages to recruit activated CD4 and CD8 T cells to the site of infection (Mohan et al., 2001). In turn, IFN-γ production is induced and further activates the macrophages (Flynn, 2006). Mycobactericidal effects are mainly triggered by activated macrophages and by cytotoxic function of T cells. The killing effect is also modulated by TNF-α inducing apoptosis of infected macrophages. Thus, the balance of pro- and anti-inflammatory cytokines fine tune the cells communication which in turn regulate the immune response (Romero-Adrian et al., 2015).

Activated macrophages are well equipped with several proteolytic enzymes in the phagolysosome to destroy the microbes (Connelly et al., 2003). In addition, activated macrophages convert molecular oxygen into reactive oxygen species (ROS), which act as highly reactive oxidizing agents that promote bactericidal effects on the infected cells (Alderton et al., 2001) . Other important metabolites produced by activated macrophages are reactive nitrogen intermediates (RNI), mainly nitric oxide (NO). Production of NO is catalyzed by the expression of an enzyme called inducible nitric oxide synthase (iNOS), induced in various cells including macrophages, neutrophils and epithelial cells (Bosca et al., 2005).

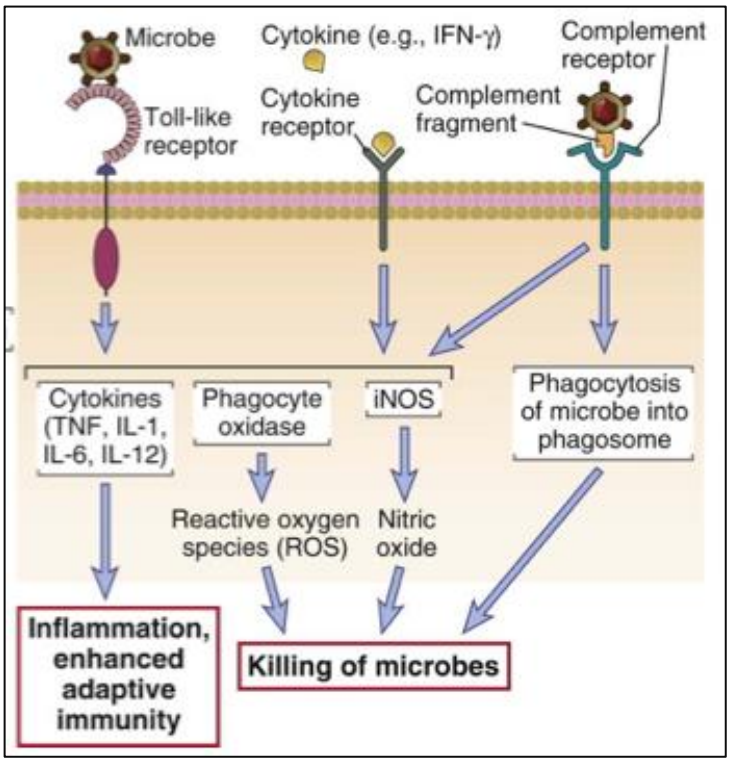

Figure 1.7: Effector functions of macrophages (Abbas et al., 2007).

NO is mainly produced in response to the microbial products that activate TLRs, especially in combination with cytokines and chemokines (Nathan and Shiloh, 2000). It has been well described that stimulation of iNOS is positively regulated by Th1 cytokines (IFN-γ, TNF-α), and negatively regulated by Th2 cytokines (IL-10, IL-4) (Figure 1.8) (Sharma et al., 2004; Roy et al., 2014). iNOS catalyzes the conversion of arginine to citrulline, which results in the production of diffusible NO. Within phagolysosomes, the increase in RNIs is generated by the reaction of NO with O_2^- which is capable to kill the microbes. The killing mechanisms are modulated by NO and RNI through the modification of bacterial DNA, proteins and lipids in both the pathogen and infected host cells (Chan et al., 1995).

During infection with *M. tuberculosis*, iNOS knock-out and immunodeficient mice are at significantly higher risk of dissemination and had lower survival rate as compared to control mice (MacMicking et al., 1997). Contrasting data have been reported regarding the role of NO and iNOS in the killing and inhibition of *M. tuberculosis* in mice model versus humans. Evidences indicated that early inhibition of mycobacterial growth by human alveolar macrophages is independent of NO production (Bingisser and Holt, 2001; Davis et al., 2007). Nevertheless, several reports described a role for NO in human host responses against *M. tuberculosis*. Studies have demonstrated that alveolar macrophages modulates the mycobactericidal effects dependent on iNOS expression (Nozaki et al., 1997).

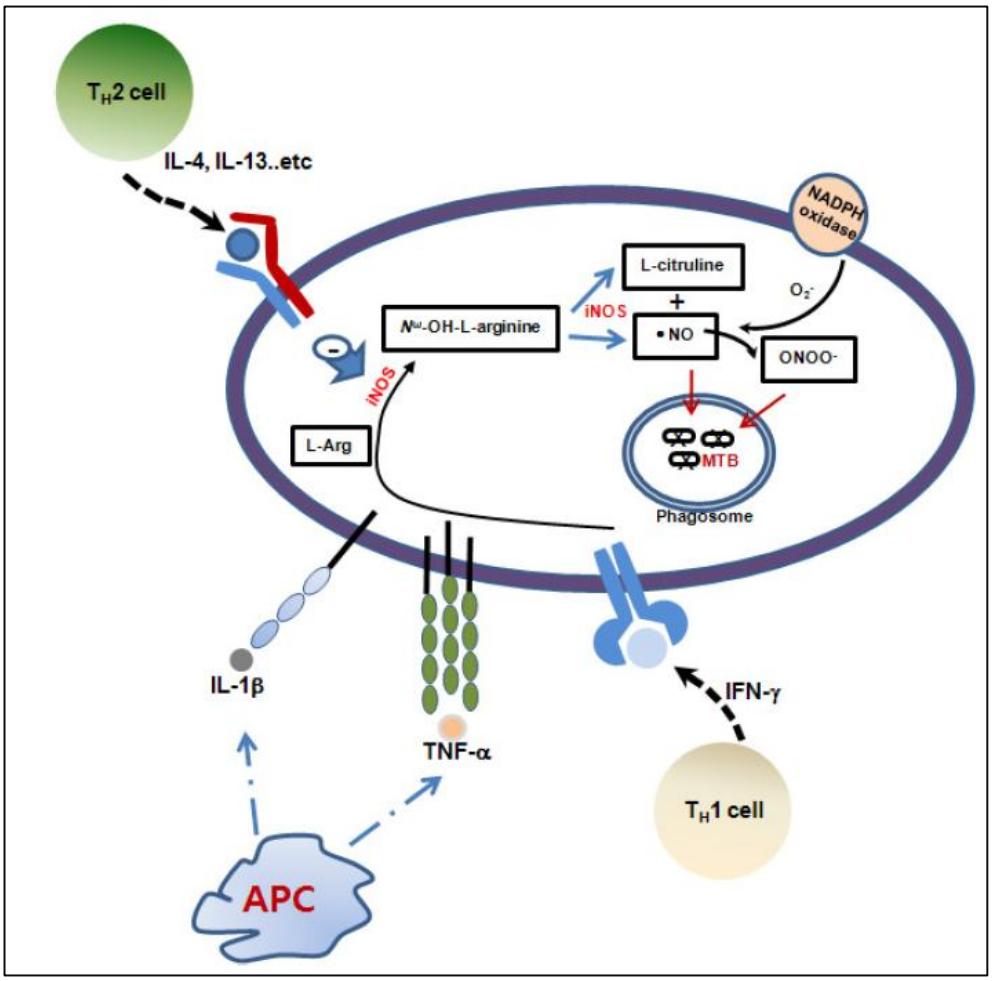

Figure 1.8: Mechanism of regulation and anti-mycobacterial function of NO. (Yang et al., 2009).